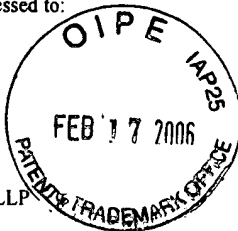


I hereby certify that this correspondence is enclosed in an envelope being deposited with the United States Postal Service as first class mail under 37 CFR 1.10 on the date indicated above and is addressed to:

PATENT  
Attorney Docket No.: 015280-290100US  
Client Ref. No.: E-171-96/0

Assistant Commissioner for Patents  
Art Unit 1644  
Washington, D.C. 20231.



TOWNSEND AND TOWNSEND AND CREW LLP

On: August 27, 2001

By: H. Munler  
H. Munler

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

KLIMPEL *et al.*

Application No.: 08/937,276

Filed: September 15, 1997

For: TARGETING ANTIGENS TO THE  
MHC CLASS I PROCESSING PATHWAY  
WITH AN ANTHRAX TOXIN FUSION  
PROTEIN

Examiner: Ronald B. Schwadron, Ph.D.

Art Unit: 1644

DECLARATION OF DR. JAY A.  
BERZOFKY UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Jay A. Berzofsky, M.D., Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I am the Chief of the Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health. I have been in this position since 1987. I am currently the Chair for the Vaccine Working Group for the National Cancer Institute.

3. I received a bachelor's degree in chemistry, summa cum laude, from Harvard University in 1967. I received a Ph.D. and an M.D. degree from the Albert Einstein School of Medicine in 1972 and 1973, respectively. I trained in medicine at Massachusetts General Hospital, and as a postdoctoral fellow in the laboratory of Nobel prize winner Dr. Christian B. Anfinsen at the National Institutes of Health. Prior to 1987, I was a Senior Investigator at the National Institutes of Health. I am also a past president of the American Society for Clinical Investigation. I am an author of 330 published or in press scientific papers and books. In addition, I am an author of chapters in several major immunology textbooks. A copy of my curriculum vitae and bibliography is attached hereto as Exhibit A.

4. The present invention relates to the surprising discovery that the anthrax toxin system can be used to deliver full length proteins to the cell cytosol for processing and presentation by MHC class I molecules to cytotoxic T cells (CTLs). In particular, the invention provides the first evidence that an anthrax toxin fusion protein comprising a full length protein-anthrax lethal factor (LF) fusion bound to anthrax protective antigen (PA) is translocated into the cell, and that the full length protein is efficiently processed into multiple epitopes by the MHC class I processing pathway and presented by MHC class I molecules to CTLs. The use of full length protein has the advantage of providing multiple epitopes that are recognized by more than one MHC class I allele. Moreover, the anthrax toxin fusion proteins of the present invention, comprising the full length protein, are more potent for sensitizing CTLs than corresponding peptide epitopes.

5. I am a named inventor on the above-referenced patent application. I have read and am familiar with the contents of this patent application. In addition, I have read the Office Action, dated February 27, 2001, received in the present case. It is my

understanding that the Examiner is concerned that the claims are obvious over Milne *et al.*, Arora *et al.*, or Leppla *et al.* in view of Donnolly *et al.* and EP 0 532 090. In particular, the Examiner asserts that it would have been obvious to one of ordinary skill in the art at the time of the invention to use an anthrax toxin fusion protein to deliver a protein to a cell, as taught by Milne *et al.*, Leppla *et al.*, or Arora *et al.*, for processing of the protein via the cytosolic MHC class I pathway and induction of a CTL response to the protein, as taught in Donnelly *et al.*

6. This declaration is provided to demonstrate that, at the time of the invention, one of skill in the art would not have had a reasonable expectation of success using the anthrax toxin fusion proteins of Milne *et al.*, Arora *et al.*, or Leppla *et al.* in combination with the methods of inducing an immune response described by Donnolly *et al.* and EP 0 532 090. Furthermore, one of skill in the art would not have been motivated to substitute an anthrax toxin fusion protein for the *Pseudomonas* toxin (PE) fusion protein used in the methods of Donnolly *et al.* and EP 0 532 090 to practice the invention. Moreover, this declaration is provided to demonstrate that, using the anthrax toxin fusion proteins of the present invention to induce an immune response, the inventors achieved results that are more effective than and superior to those of the closest prior art.

7. In my scientific opinion, Milne *et al.*, Arora *et al.*, and Leppla *et al.* teach methods for cellular delivery of small fragments of proteins or peptide epitopes, rather than full length proteins, using anthrax toxins comprising PA and an LF fusion protein. Anthrax lethal toxin is a binary bacterial toxin comprising two proteins, LF and PA. PA binds to a cellular receptor and is cleaved, revealing an LF binding site. LF binds to PA, forming anthrax toxin, which is translocated into the cell. However, as described in detail below, these references fail to teach the use of anthrax toxin fusion protein to elicit a T cell response, by delivering proteins or peptides to a cytosolic MHC class I pathway for epitope processing and cell surface presentation.

For example, Milne *et al.* teaches compositions where the catalytic domain of diphtheria toxin A chain is fused to LF and translocated into a cell, via PA binding. Arora *et al.* teaches compositions where the catalytic domain of *Pseudomonas* exotoxin A is fused to LF and translocated into a cell, via PA binding. Leppla *et al.* teaches generic compositions where peptides or protein fragments are fused to LF for cellular translocation, via PA binding.

Each of these references therefore discloses methods of internalizing protein fragments or peptide epitopes, for delivery of an activity to a cell, e.g., a cytotoxic activity. None of these references teach that anthrax toxin can be used to deliver a protein to a cytosolic MHC class I processing pathway, for induction of an immune response.

Furthermore, the teachings of Milne *et al.*, Arora *et al.*, and Leppla *et al.* at most only propose the use of anthrax toxins to deliver full length proteins to cells. Also, Arora *et al.* at most only proposes that anthrax toxin could be used to present peptides to MHC class I molecules. However, as described below, the art teaches away from the use of bacterial toxins to present peptides to MHC class I molecules, let alone full length proteins. These teachings therefore do not provide any indication as to what parameters are critical, nor provide any direction as to which of the many possible choices for making and using anthrax toxins for delivery of full length proteins to the cytosol for processing and presentation by MHC class I molecules to CTLs. None of these references would provide one of skill in the art with a reasonable expectation of success for practicing the claimed invention.

8. In my scientific opinion, Donnelly *et al.* and EP 0 532 090 teach methods of inducing an immune response using *Pseudomonas* exotoxin ("PE") fused to a peptide epitope (*i.e.*, peptide epitopes from influenza A matrix protein or nucleoprotein). PE is a single subunit bacterial toxin, rather than a binary toxin like anthrax. These references therefore teach neither the use of binary toxins nor the use of full length proteins to induce an immune response. The references merely describe attempts to use such PE fusion proteins to translocate peptide epitopes into a cell for presentation by the MHC class I pathway.

Moreover, the same authors in a separate publication by Ulmer *et al.* (further discussed below) demonstrate that PE toxin fusion proteins are likely processed via an alternative, endosomal processing pathway that is less efficient than the MHC class I cytosolic pathway. Therefore, one skilled in the art would conclude that such bacterial toxin fusion proteins do not deliver antigen to the MHC class I cytosolic pathway. This reference thus teaches away from using bacterial toxin fusion proteins for processing of an antigen and presentation of an antigen by MHC class I molecules.

In addition, these references teach the administration of a 12-mer amino acid epitope fused to PE toxin. Due to its size, such an epitope does not require cellular processing in order to be displayed by MHC class I molecules. Prior to the time of the present invention, the disadvantages associated with the translocation of large proteins (*e.g.*, inefficient translocation) and the advantages associated with the use of peptide epitopes (*e.g.*, efficient translocation and generation of T cells directed to a single epitope) were known to one of skill

in the art. Consistent with this understanding, Donnelly *et al.* and EP 0 532 090 teach the use of bacterial toxin fusion proteins encoding peptide epitopes, and not full length proteins. These references therefore fail to provide the requisite motivation to one of skill in the art to use anthrax toxin fusion proteins for delivery of a full length protein to a cell for processing and presentation by MHC class I.

9. The present application provides the first evidence that a bacterial toxin system (anthrax toxin) can be used to introduce a full length protein into the cytosol for processing via the MHC class I pathway and presentation by MHC class I molecules to CTLs. This processing of the full length protein and presentation to CTLs, via the MHC class I pathway, was demonstrated by treating antigen-presenting cells with lactacystin, which inhibits proteasome function required for cytosolic MHC class I processing (*see* specification, page 3, lines 7-11). In addition, the evidence demonstrates that the anthrax toxins of the present invention, fused to full length proteins, are more potent for sensitizing CTLs than a peptide epitope from the same full length protein (*see* Goltez *et al.*, *PNAS USA* 94:12059-12064 (1997), page 12061, previously submitted on May 24, 1999 as Appendix A).

10. The present inventors are therefore the first to show that a full length protein, fused to LF and translocated into a cell by anthrax toxin, is processed by the cytosolic MHC class I pathway and presented by MHC class I molecules to CTLs. In addition, the fusion proteins of the present invention are more potent for sensitizing CTLs than peptide epitopes and thus provide superior results over that taught in the prior art. In contrast, the prior art describes only the use of bacterial toxins to translocate protein fragments or epitopes into cells, for delivery of, e.g., a cytotoxic activity. In addition, the prior art teaches that bacterial toxin fusion proteins are likely processed by an alternative endocytic processing pathway, and not the cytosolic MHC class I processing pathway that results in the efficient presentation of a processed protein by MHC class I molecules to CTLs. Thus, the prior art teaches away from the claimed invention. But, even if one of skill in the art was motivated to try this approach, undue experimentation would be required to practice the claimed invention in view of the teachings of the prior art. Therefore, it is my scientific opinion that these prior studies would have provided neither motivation nor a reasonable expectation of success to use anthrax toxin to introduce a full length protein into a cell and elicit a CTL immune response, by processing the full length protein via the cytosolic MHC class I pathway, and presenting the resulting

epitopes by MHC class I molecules. Moreover, in view of the prior art teachings, our results are unexpected and surprisingly effective.

11. In the Office Action, the Examiner asserts that “the ability of the protein-LF PA binary complex to translocate the protein into the cytosolic MHC class I processing pathway is an inherent property of the complex.” I disagree with the Examiner’s assertion for the following reasons. Prior to the time of the invention, such properties of anthrax toxin proteins would have been unpredictable and unexpected. At that time, the prior art taught that peptide epitopes delivered to a cell using bacterial fusion toxin proteins likely bind to preexisting MHC class I molecules that are recycled and internalized via an endocytic pathway, rather than being processed by the more efficient cytosolic MHC class I pathway. The prior art provides no information regarding processing of bacterial toxin fusion proteins via the cytosolic MHC class I pathway. Moreover, the prior art taught that large proteins were inefficiently translocated into the cell, if at all. The present inventors are the first to demonstrate that anthrax toxins can be used to deliver whole proteins to the cell cytosol for processing and presentation by MHC class I molecules to CTLs. Prior to this discovery, such properties and uses of bacterial toxin proteins for delivery of whole proteins to cells would have been unpredictable and unexpected.

12. The Examiner further asserts that Arora *et al.* teach that anthrax toxin fusion proteins are translocated into the cell and induce a CTL response to the heterologous protein fused to LF; and that the teachings of Ulmer *et al.* support the Examiner’s assertions. For the following reasons, I disagree with the Examiner’s assertion.

As mentioned above, the teachings of Arora *et al.* are directed to cellular delivery of small fragments of proteins or peptide epitopes and at most only propose the use of whole proteins. Arora *et al.* do not provide any indication as to what parameters are critical, nor provide any direction as to which of the many possible choices for making and using anthrax toxin fusion proteins for delivery of whole proteins to the cytosol for processing via the MHC class I pathway and presentation by MHC class I molecules to CTLs.

Moreover, the teachings of Ulmer *et al.* relied on by the Examiner actually - teach away from the claimed invention. The conclusion of the Ulmer *et al.* study is that the PE

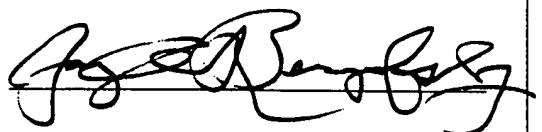
fusion protein is not translocated into the cytosolic MHC class I pathway, but rather enters an endosomal pathway. This reference therefore teaches away from the use of bacterial toxin fusion proteins for introducing whole proteins into the cytosolic MHC class I pathway for subsequent processing and presentation by MHC class I molecules.

Further, the Examiner points out that such experiments by Ulmer *et al.* "do not exclude that under physiological conditions . . . translocation into the cytosolic MHC class I processing does not occur" (emphasis added). Merely teaching that the experiments do not exclude what is not known fails to provide support or a rational basis for concluding that the prior art teaches or suggests the translocation of bacterial toxin fusion proteins into the cytosolic MHC class I processing pathway.

Therefore, the teachings of Ulmer *et al.* do not support the Examiner's assertion that Arora *et al.* teaches the use of anthrax toxin fusion proteins for delivery of whole proteins to the cytosol for processing by the MHC class I processing pathway and presentation by MHC class I molecules to CTLs.

13. In conclusion, the present application provides the first evidence that full length proteins fused to anthrax toxin are translocated into the cell, enter the cytosolic MHC class I processing pathway, and are subsequently presented by MHC class I molecules to CTLs. This evidence is contrary to the teachings of the prior art, which suggest that bacterial toxin fusion proteins are likely processed via an endocytic pathway. Moreover, the anthrax toxin fusion proteins of the present invention, comprising a full length protein, are more potent for sensitizing CTLs than peptide epitopes. Thus, using the anthrax toxin fusion proteins of the present invention, the inventors achieved results that are more effective than and superior to those of the closest prior art. In view of the prior art, these results are unobvious and unexpected, because the prior art teaches the advantages and use of small fragments of proteins and peptide epitopes over full length proteins, and teaches disadvantages associated with the translocation of full length proteins. Thus, it is my scientific opinion that the teachings of Milne *et al.*, Arora *et al.*, or Leppla *et al.* in view of Donnolly *et al.* and EP 0 532 090 do not provide one of skill in the art with a reasonable expectation of success, nor motivate one of skill in the art to practice the invention.

- Date: 24 August, 2001

By: 

## CURRICULUM VITAE

Name: Jay Arthur Berzofsky

Date and Place of Birth: April 13, 1946, Baltimore, Maryland

Marital Status: Married to Sharon M. Miller, June 27, 1969; two children  
Alexander, April 30, 1974, and Marcus, May 27, 1976

Education:

1967 - A.B., Harvard University (Summa Cum Laude in Chemistry)  
1971 - Ph.D., Albert Einstein College of Medicine, Molecular Biology  
1973 - M.D., Albert Einstein College of Medicine, Medical Scientist  
Training Program

Brief Chronology of Employment:

1973 - 1974	Medical Internship (Straight Medicine), Massachusetts General Hospital, Boston, Massachusetts
1974 - 1976	Research Associateship, Laboratory of Chemical Biology National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health
1976 - 1979	Investigator ("Expert"), Metabolism Branch, National Cancer Institute, National Institutes of Health
1979 - 1987	Senior Investigator, Metabolism Branch, National Cancer Institute, National Institutes of Health
1987 - Date	Chief, Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, National Institutes of Health

Honors/Awards:

Detur Prize, Harvard University, 1964  
Harvard College Scholarship, Harvard University, 1964  
Phi Beta Kappa, Junior Year, Harvard University, 1966  
Summa Cum Laude in Chemistry, Harvard University, 1967  
Sophia Freund Prize for Graduate with Highest Cumulative Grade Point  
Average, Harvard College, 1967  
NIH Special Achievement Award, 1982  
Hollister - Stier's Distinguished Lectureship, Washington State  
University, 1986  
J. W. McLaughlin Fund Distinguished Contributions to Immunology Lectureship,  
University of Texas Medical School, Galveston, 1987



U. S. Public Health Service Superior Service Award, 1988  
 31st Michael Heidelberger Award and Lecture, Columbia University, 1992  
 McLaughlin Visiting Professorship, University of Texas Medical School,  
 Galveston, 1992  
 American Society for Clinical Investigation, President 1993-94  
 Fellow of the American Association for the Advancement of Science, 1995  
 Cytokine Interest Group Best Paper of 2000 Award to fellow in lab, 2001

Professional Society Memberships:

Association of Harvard Chemists, 1967 - present  
 New York Academy of Sciences, 1971 - present  
 American Association of Immunologists, 1977 - present  
 Undersea Medical Society, 1978 - 1988  
 American Federation for Clinical Research, 1979 - present  
 American Society of Biological Chemists, 1980 - present  
 American Society for Clinical Investigation, 1983 - present,  
 Secretary-Treasurer, 1989 - 1992  
 President-elect, 1992-1993  
 President, 1993-94  
 Association of American Physicians, 1990 - present

Editorial Positions:

Associate Editor, *Journal of Immunology*, 1980 - 1984  
 Editorial Advisory Board, *Journal of Molecular and Cellular Immunology*, 1983 -  
 present  
 Advisory Editor, *Molecular Immunology*, 1985 - 1988  
 Editorial Board, *Peptide Research*, 1987 - present  
 Transmitting Editor, *International Immunology*, 1988 - present  
 Editorial Board, *Journal of Human Virology*, 1997-present  
 Consulting Editor, *Journal of Clinical Investigation*, 1998-present  
 Section Editor, *Journal of Clinical Immunology*, 2002-present

Professional Committees:

American Association of Immunologists, Membership Committee, 1981 - 1988  
 American Association of Immunologists, Chairman of Membership Committee,  
 1983 - 1988  
 NIH Clinical Center Compensable Events Committee, 1982 - present  
 American Society for Clinical Investigation, Council, 1989-1994  
 NCI Division of Clinical Sciences Promotion and Tenure Committee, 1995-  
 present.  
 NCI Division of Clinical Sciences Research Advisory Group, 1995-present  
 NCI Director's Intramural Advisory Board, 1997-99  
 NIH AIDS Vaccine Research Center Steering Committee, 1997-present  
 NIH Search Committee for Director of Office of AIDS Research, 1997-98  
 NIAID Malaria Vaccine Task Force, 1998-present  
 NCI Vaccine Working Group, Chairman/Organizer, 1998-present  
 NCI/CCR Immunology Faculty Steering Committee, 2001-present  
 NCI/CCR HIV & Virology Faculty Steering Committee, 2001-present

Military Service:

Commissioned Corps, United States Public Health Service, 1974 - 1976

Other Research Experience:

Summers, 1962 - 1965      Research Assistant, Pediatric Research Unit (H. M. Nitowsky), Sinai Hospital, Baltimore, Maryland

Summer, 1966 Research Assistant, Organic Synthesis Laboratory  
(C. H. Robinson), Department of Pharmacology, Johns  
Hopkins School of Medicine, Baltimore, Maryland

Summer, 1967 Visiting Scientist, Laboratoire d'Enzymologie (G. N. Cohen), Centre National de la Recherche Scientifique, Gif-sur-Yvette, France

Medical Licensure: Maryland and Massachusetts

Major Outside Activities

Medimmune, Inc.—Scientific Founder and Chair, Scientific Advisory Board, 1989-present  
 Magainin Pharmaceuticals, Inc.—Member, Scientific Advisory Board, 1991-97  
 Diacrin, Inc.—Member, Scientific Advisory Board, 1993-present  
 Pharmadyne, Inc.—Scientific Co-Founder and Chair, Scientific Advisory Board, 1997-present  
 Boston University Community Technology Fund—Consultant, 1997-present  
 Health Care Ventures, Inc.—consultant, 1998-present  
 EMD Pharmaceuticals, Inc.—consultant, 2000-present  
 Epivax, Inc.—Member, Scientific Advisory Board, 2000-present

Major areas of research:

1. Molecular basis of antigen recognition by T lymphocytes
2. Processing of antigens and their presentation by major histocompatibility molecules
3. Structure of antigenic sites on protein antigens
4. Genetic regulation of the immune response
5. Design and development of artificial vaccines based on immunological principles and peptide synthesis or recombinant DNA technology
6. AIDS vaccines and diagnostic techniques
7. Malaria vaccines
8. Cancer vaccines
9. Antigen-antibody interactions
10. Structure-function relationships in proteins and protein conformation.
11. Regulation of tumor immunosurveillance and T cell function by cytokines
12. Mucosal immunity and vaccines

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Jay Arthur Berzofsky

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## Presentation of an exogenous antigen by major histocompatibility complex class I molecules

Cytotoxic T lymphocytes (CTL) generally recognize peptides derived from endogenously expressed proteins in association with nascent major histocompatibility complex (MHC) class I molecules. In contrast, peptides derived from exogenous proteins associate with MHC class II following endocytosis to an endosomal compartment. However, we have recently demonstrated that exogenous fusion proteins consisting of the binding and translocating domains of *Pseudomonas* exotoxin (PE) fused with CTL epitopes derived from either influenza matrix protein (PEMa) or nucleoprotein are internalized, processed, targeted to and presented by MHC class I (Donnelly et al. 1993, Proc. Natl. Acad. Sci. USA 1993. 90: 3530). PE is known to be internalized, processed in endosomes, and translocated to the cytosol during intoxication of cells. However, our present studies demonstrate that, unlike PE, PEMa does not require translocation to the cytosol to exert its effect. First, two inhibitors of PE toxicity that exert their effects at steps subsequent to endosomal processing had no effect on the sensitization of target cells for CTL-mediated lysis by PEMa. NH<sub>4</sub>Cl, which inhibits PE by raising endosomal pH, and brefeldin A, which inhibits PE by disrupting the Golgi complex, did not inhibit sensitization of target cells by PEMa. Second, PEMa was capable of sensitizing for lysis T2 mutant cells, which are defective in transport of peptides from the cytosol to the lumen of the endoplasmic reticulum for presentation by MHC class I. These results suggest that PEMa is proteolytically processed in endosomes, and association with MHC class I does not require nascent MHC molecules. Such a process may involve internalized MHC class I, and subsequent expression of the peptide-MHC complexes on the cell surface would then lead to recognition by CTL.

### 1 Introduction

Cytotoxic T lymphocyte (CTL) responses are generated by presentation of antigens to CD8<sup>+</sup> T lymphocytes by MHC class I molecules. A key to this process is the intracytosolic presence of antigens, such as in the case of endogenously synthesized viral proteins. Because CTL are important for protection against viral infections [1-4] and tumors [5] and because CTL are able to recognize epitopes derived from viral proteins, it is desirable to develop vaccines designed to engender a CTL response. Such a response has been generated *in vivo* using plasmid DNA [6], or retroviral vectors or modified pathogens [7-9] to express foreign proteins *in situ*. Alternatively, exogenous proteins and peptides have been delivered intracellularly for presentation by MHC class I, mediated by immunostimulating complexes [10], saponin adjuvants [11], synthetic lipopeptides [12], and pH-sensitive liposomes [13]. Recently, we described a recombinant protein consisting of the binding and translocating domains of *Pseudomonas* exotoxin A (PE) fused with the HLA-A2.1-restricted CTL epitope of

influenza matrix protein [14]. The native toxin is internalized by cell surface proteins, undergoes intracellular processing, and translocates its catalytic domain to the cytosol [15, 16]. The PE fusion protein (PEMa) was internalized and processed for presentation by MHC class I in target cells of the appropriate haplotype. The initial phase of intracellular trafficking of PEMa was shown to be similar to that of the toxin, in that a mutant of PEMa corresponding to a point mutation in PE that inhibits endosomal processing was inhibitory for sensitization of cells for CTL-mediated lysis by PEMa. In the present study, the intracellular trafficking of PEMa was further characterized. Surprisingly, delivery of PEMa to the cytosol was not required for presentation of the matrix epitope by MHC class I molecules. The implications of these findings are discussed in the context of current knowledge concerning antigen presentation by MHC class I.

### 2 Materials and methods

#### 2.1 Materials

T2 and T1 cells were obtained from Peter Cresswell (Yale University); a mouse monoclonal antibody to HLA-A2.1 and HMY-A2 cells were obtained from Hans Zweerink (Merck Research Laboratories); U2-OS cells were purchased from ATCC; tissue culture supplies from Gibco; [<sup>35</sup>S] methionine (~1200 Ci/mmol; 1 Ci = 37 GBq) and <sup>51</sup>Cr K(350-600 mCi/mg) from Amersham Corporation; brefeldin A (BFA) from Epicenter Technologies; PE from List Laboratories; FITC-conjugated goat anti-mouse IgG (Fc portion) and a mouse IgG2a control serum were from Cappel-Organon Technika.

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Abbreviations: BFA: Brefeldin A ER: Endoplasmic reticulum  
PE: *Pseudomonas* exotoxin A PEMa: PE-influenza matrix epitope fusion protein

Key words: Antigen presentation / Cytotoxic T lymphocytes / Influenza

## 2.2 Cell culture

U2-OS cells were maintained in McCoy's 5A medium with 15% fetal bovine serum (FBS) and were removed from flasks with 0.1 M EDTA in phosphate-buffered saline (PBS). Human CTL specific for matrix protein were obtained from a normal HLA-A2.1<sup>+</sup> donor and were maintained *in vitro* as described by Bednarek et al. [17] with minor modifications [14]. T2, T1 and HMY-A2 cells were grown in suspension in RPMI 1640 containing 10% FBS.

## 2.3 Biosynthetic labeling

For measurement of PE toxicity, U2-OS cells were plated in a 96-well plate at a density of 20 000 cells/well and allowed to attach overnight. Cell monolayers were treated with or without BFA or  $\text{NH}_4\text{Cl}$  for 1 h at 37°C, followed by the addition of PE (0-1000 pM final concentration) for 15 min. Cells were washed with culture medium containing BFA or  $\text{NH}_4\text{Cl}$  and incubated with [ $^{35}\text{S}$ ]methionine (2  $\mu\text{Ci}$ ) and BFA or  $\text{NH}_4\text{Cl}$  for 5 h. Cells were washed with ice-cold culture medium, lysed with sodium dodecylsulfate (0.1%) and added to an equal volume of ice-cold trichloroacetic acid (20%). Incorporation of radioactivity into acid precipitable material was determined by liquid scintillation spectrometry. For measurement of protein secretion, U2-OS cell monolayers were treated with BFA (1-10  $\mu\text{g}/\text{ml}$ ) for 30 min at 37°C, followed by the addition of [ $^{35}\text{S}$ ]methionine. Aliquots of cell culture supernatant were taken at various times and spotted onto filter papers. Secreted proteins were precipitated onto the filters by soaking in ice-cold trichloroacetic acid (10%) for 1 h. Filters were washed three times in ice-cold trichloroacetic acid (5%) and three times in ice-cold ethanol, dried and radioactivity was determined as before.

## 2.4 Cytotoxic T lymphocyte assay

U2-OS cells were plated in 24-well plates at  $4 \times 10^5$  cells per well and labeled overnight with 2  $\mu\text{Ci}/\text{ml}$  of  $^{51}\text{Cr}$ . The following day, cell monolayers or suspensions were exposed to BFA (1-10  $\mu\text{g}/\text{ml}$ ) or  $\text{NH}_4\text{Cl}$  (10-80 mM) for designated periods of time. Cells were incubated with PEMa, washed, and in some cases further incubated in the absence of PEMa, and finally washed with PBS. For BFA-treated target cells, all wash solutions contained BFA. To obtain influenza virus-infected targets, cells were incubated with influenza A virus (A/Victoria/73) for 30 min, washed, and incubated for a further 2 h. EDTA (0.1 M in PBS) was then used to detach U2-OS cells from the plates, and the cells were washed twice more and resuspended in RPMI 1640 medium with 10% FBS and 0.01 M Hepes at  $5 \times 10^4$  cells/ml. T1 and T2 cells were labeled overnight with 2  $\mu\text{Ci}/\text{ml}$  of  $^{51}\text{Cr}$ , and washed by sedimentation and resuspension. Where indicated, cells were fixed with glutaraldehyde (0.1%) for 1 min on ice. Target cells were plated in triplicate at 0.1 ml per well in round-bottom, 96-well plates with 0.1 ml CTL at effector:target ratios of 30:1, 20:1 or 10:1. Cells were incubated at 37°C, sedimented, and 20  $\mu\text{l}$  of supernatant was removed for counting in an LKB 121250 scintillation counter. Results were expressed as specific lysis = [(counts released with CTL)-(counts

released without CTL)/(counts released by 6 M HCl)-(counts released without CTL)]  $\times 100$ .

In bystander killing experiments, T1, T2 and HMY-A2 cells were incubated with PEMa (1  $\mu\text{g}/\text{ml}$ ) for 30 min, washed and added to adherent U2-OS cells that were loaded overnight with  $^{51}\text{Cr}$ , as before. The cell mixtures were incubated for 2 h and the PEMa-pulsed T1, T2 or HMY-A2 cells were decanted. The U2-OS cells were washed and incubated with influenza-specific CTL for 2 h. In the converse experiment, U2-OS cells were incubated with PEMa, and T1, T2 or HMY-A2 cells were loaded with  $^{51}\text{Cr}$ , then the cell mixtures were incubated as before. The T1, T2 and HMY-A2 cells were decanted and incubated with CTL for 2 h. In each case, negative controls consisted of cells without prior incubation with PEMa, as well as no addition of CTL to bystander cells. As positive controls, each set of bystander cells was incubated with PEMa or matrix peptide (57-68) to demonstrate sensitization.

## 2.5 Immunofluorescence staining

U2-OS cells were treated with or without BFA (1-10  $\mu\text{g}/\text{ml}$ ) for various periods of time (0-10 h), and removed from culture flasks with EDTA. For staining of surface MHC class I molecules, cells were washed with PBS containing FBS (5%) and sodium azide (0.01%), then incubated with a monoclonal HLA-A2.1 antibody or a normal mouse IgG2a control (12.5  $\mu\text{g}/\text{ml}$  final concentration) for 30 min on ice. Cells were washed, incubated with a FITC-conjugated goat anti-mouse IgG (Fc portion) (25  $\mu\text{g}/\text{ml}$ ) for 30 min on ice, and washed again. Measurements were as mean channel fluorescence intensity using a Becton Dickinson FACScan flow cytometer with an excitation wavelength of 488 nm and a band pass filter of 530 nm. A minimum of 3500 events/sample were analyzed.

## 3 Results

The fusion protein PEMa has been previously shown to be internalized by U2-OS and HMY A2 target cells and presented by MHC class I [14]. These cells were chosen because they express the HLA-A2.1 MHC haplotype, in order that the HLA-A2.1-restricted CTL epitope of influenza matrix protein (amino acids 57-68) would be presented by MHC class I and specifically recognized by appropriate CTL.

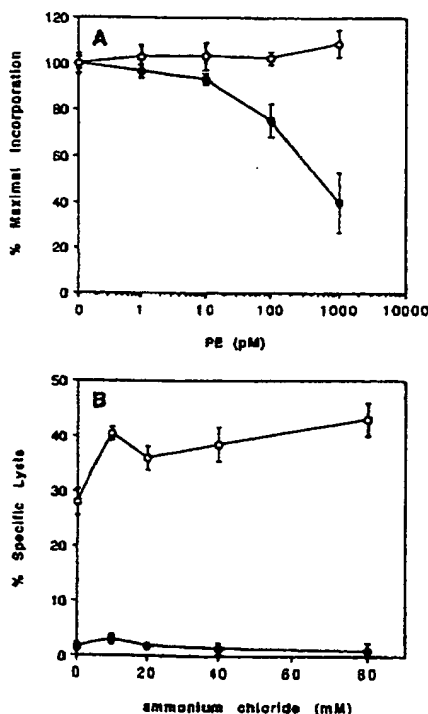
Additionally, these cells bind and process PE, and are sensitive to the toxin (unpublished observations). It was demonstrated that, as in the case of PE, delivery to endosomes and proteolytic processing of PEMa therein was a necessary step in its action. In the present study, we sought to characterize further the intracellular trafficking of PEMa in order to investigate the location of association with MHC class I during sensitization of target cells for lysis by CTL. This was accomplished using compounds that have been shown to block different steps in the processing of PE.

PE intoxication is known to be inhibited by weak amines, such as ammonium chloride, at a step subsequent to endosomal proteolytic processing [16]. The precise mechanism of inhibition by primary amines has not yet been

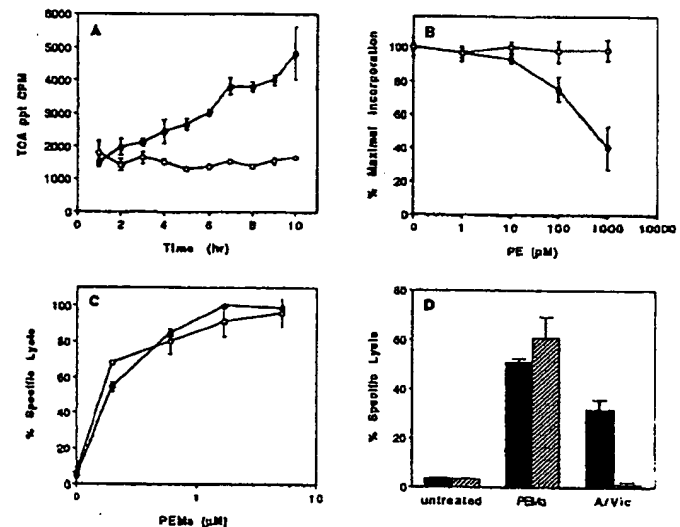
defined. But, given that PE may translocate from some other compartment such as the ER [18, 19], there are several potential events following endosomal processing that could be affected (e.g., budding of vesicles from endosomes or Golgi, binding to the KDEL receptor, or translocation to the cytosol). U2-OS cells were completely insensitive to the toxin in the presence of ammonium chloride at concentrations shown to inhibit PE in other cell types (Fig. 1 A). Under these conditions, however, sensitization of cells by PEMa for CTL-mediated lysis was not affected (Fig. 1 B). These results indicate that an obligatory step subsequent to endosomal processing of the toxin is not necessary for sensitization by PEMa and represents a divergence in the pathways of the two molecules.

Recently, BFA was shown to inhibit intoxication of cells by PE [20, 21]. Although the means of this inhibition is not fully understood, BFA treatment is known to cause: (i) a cessation in protein exocytosis due to the disruption and disappearance of the Golgi complex [22, 23], and (ii) morphologic but not functional changes in endosomes [24]. Due to the presence of a KDEL-like sequence on the C-terminus of PE that is necessary for PE toxicity [18], it

has been proposed that prior to translocation to the cytosol PE is transported from endosomes to the Golgi complex and endoplasmic reticulum (ER) in a process that involves the KDEL receptor [19]. Therefore, inhibition of PE toxicity by BFA is likely to be due to its effects on the Golgi complex and, hence, may prevent trafficking of PE to the ER for translocation to the cytosol. Treatment of U2-OS cells with BFA resulted in an inhibition of protein secretion (Fig. 2 A), and also prevented intoxication of the cells by PE (Fig. 2 B). Incubation of the cells with BFA did not have a discernible effect on sensitization by PEMa (Fig. 2 C), demonstrating that PEMa does not require delivery to, or passage through the Golgi complex, for association with MHC class I. As a positive control for the inhibitory effect of BFA on antigen presentation by nascent MHC class I, virus-infected U2-OS cells were tested. Virus-infected cells were sensitized for lysis by CTL, but not when the cells were treated with BFA following a 30-min pulse with virus (Fig. 2 D). Since evidence indicates that association of



**Figure 1.** Effect of ammonium chloride on PE toxicity and sensitization by PEMa. Panel A: PE toxicity. U2-OS cells were incubated in the presence (open circles) or absence (closed circles) of  $\text{NH}_4\text{Cl}$  (30 mM) and PE toxicity was measured as the inhibition of incorporation of  $[^{35}\text{S}]$  methionine, as detailed in Sect. 2.3. The data are plotted as incorporation of radioactivity into acid-precipitable material, as a percentage of maximal incorporation, versus PE concentration. Error bars represent  $\pm$  SD, where  $n = 6$ . Panel B: Sensitization by PEMa. Cells were incubated with  $\text{NH}_4\text{Cl}$  at the indicated concentrations for 2 h at  $37^\circ\text{C}$ , followed by the addition of PEMa (open circles) at  $1 \mu\text{M}$  for 2 h. Controls were incubated in the absence of PEMa (closed circles). Cells were fixed and incubated with CTL (effector: target = 20:1) for 2 h at  $37^\circ\text{C}$ . CTL-mediated lysis of target cells is shown as % specific lysis versus  $\text{NH}_4\text{Cl}$  concentration. Error bars represent  $\pm$  SD, where  $n = 3$ .



**Figure 2.** Effect of BFA on U2-OS cells. Panel A: Protein secretion. Cells were incubated with (open circles) or without (closed circles) BFA ( $1 \mu\text{g}/\text{ml}$ ) and protein secretion into the culture supernatant was measured. Shown is acid-precipitable radioactivity versus time in BFA. Similar results were obtained with  $10 \mu\text{g}$  BFA (not shown). Error bars represent  $\pm$  SD, where  $n = 3$ . Panel B: PE toxicity. Cells were incubated with (open circles) or without (closed circles) BFA ( $1 \mu\text{g}/\text{ml}$ ) and effect of PE on protein synthesis was measured as incorporation of  $[^{35}\text{S}]$  methionine. Shown is acid-precipitable radioactivity in the cells, as a percentage of maximal incorporation, versus PE concentration. Error bars represent  $\pm$  SD, where  $n = 6$ . Panel C: Sensitization by PEMa. Cells were incubated with (open circles) or without (closed circles) BFA ( $2 \mu\text{g}/\text{ml}$ ) for 2 h at  $37^\circ\text{C}$ , followed by the addition of PEMa ( $1 \mu\text{M}$ ) for 4 h. The cells were washed with culture medium containing BFA and incubated with CTL at an effector: target ratio of 20:1 for an additional 1.5 h in the presence of BFA. At this concentration, BFA had no detrimental effect on the ability of CTL to lyse target cells (not shown). Results are plotted as % specific lysis versus PEMa concentration. Error bars represent  $\pm$  SD, where  $n = 3$ . Panel D: Sensitization by virus infection. Cells were: (i) untreated, (ii) incubated with PEMa ( $10 \mu\text{M}$ ) in the presence or absence of BFA, or (iii) incubated with influenza A virus (A/Victoria/73), for 30 min and washed. The cells were incubated a further 2 h in the presence (striped bars) or absence (solid bars) of BFA ( $2 \mu\text{g}/\text{ml}$ ) and finally with CTL (25:1 E:T) for 2 h. Error bars represent  $\pm$  SD, where  $n = 3$ .

peptides with nascent MHC class I occurs in the ER [25, 26], it is unlikely that the influenza matrix epitope from PEMA associated with nascent MHC class I. It is possible, though, that internalized PEMA could have intersected with nascent MHC class I in a compartment that is spared by BFA treatment, such as the *trans* Golgi network (TGN), during transit to the cell surface. However, it was recently shown that BFA inhibits the constitutive and regulated secretion of proteins from the TGN to the plasmalemma [27].

Therefore, MHC class I and influenza matrix peptides, if present together in the TGN during BFA treatment, would be trapped and not reach the cell surface. Moreover, sensitization of cells by PEMA was observed after prolonged treatment of cells with BFA (10 h) (data not shown), which based on intracellular transport of MHC class I molecules in other cells [28, 29] should have been sufficient time to clear the transport vesicles containing newly synthesized MHC class I operating between the TGN and cell surface. Therefore, it is more likely that association of the matrix epitope derived from PEMA occurred in an intracellular compartment containing preexisting MHC class I molecules. It is perhaps not surprising that BFA did not inhibit sensitization by PEMA since PE fusion proteins were capable of sensitizing target cells for lysis whether or not the KDEL-like sequence was present on the C-terminus [14]. Sensitization by a PE fusion protein lacking a KDEL-like sequence suggests that transport to the ER by the KDEL receptor is not required for delivery to MHC class I and further supports the conclusion that nascent MHC class I molecules are not involved.

To test this hypothesis further, T2 mutant cells were used. These cells contain a deletion in the MHC region of the genome [30, 31] and are defective in the transport of peptides from the cytosol to the lumen of the ER for association with MHC class I [32]. Yet, these cells express HLA-A2 molecules on the cell surface due to presentation of peptides derived from the signal sequences of endogenous proteins [33]. Therefore, if delivery of PEMA to the cytosol is a necessary step prior to association with MHC class I, PEMA would not be capable of sensitizing T2 cells for lysis by CTL. In fact, PEMA sensitized T2 cells with the same or better efficiency as wild-type T1 cells (Fig. 3).

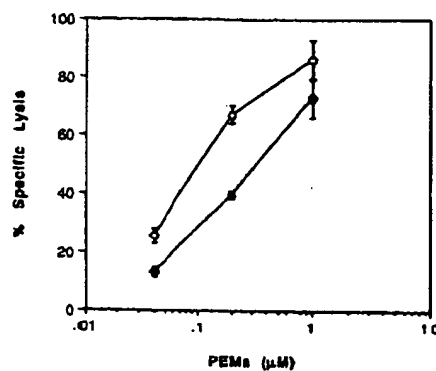


Figure 3. Sensitization of T2 cells by PEMA. T1 (closed circles) and T2 (open circles) cells were incubated with PEMA for 15 min at 37°C, washed and incubated for a further 3 h. CTL were added at an effector:target ratio of 10:1 for 3 h at 37°C. Shown is % specific lysis of target cells versus PEMA concentration. Error bars represent  $\pm$  SD, where  $n = 3$ .

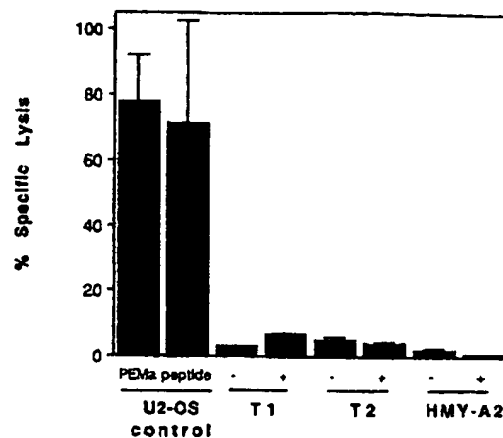
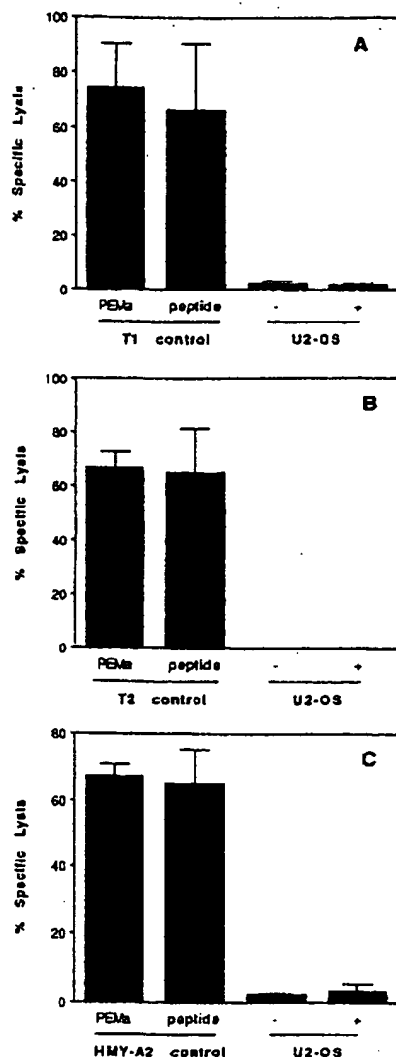


Figure 4. Lack of sensitization of U2-OS cells for lysis by excretion of peptide by antigen presenting cells. U2-OS cells served as bystander cells and were loaded with  $^{51}\text{Cr}$  overnight. T1, T2 and HMY-A2 cells served as antigen presenting cells and were incubated with (+) or without (-) PEMA (1  $\mu\text{g}/\text{ml}$ ) for 30 min and washed. The different cell mixtures were incubated together, separated and further incubated with influenza-specific CTL as described in Sect. 2.4. As positive controls, U2-OS cells were incubated with PEMA or matrix peptide (1  $\mu\text{g}/\text{ml}$ ) for 2 h, then incubated with CTL. Error bars represent  $\pm$  SD, where  $n = 3$ .

These results suggest that PEMA is internalized to an intracellular compartment other than the cytosol, Golgi complex or ER where it associates with MHC class I for eventual presentation on the cell surface.

To address the possibility that sensitization of target cells with PEMA was accomplished by internalization and processing of PEMA followed by excretion of peptide for association with cell surface MHC class I, bystander killing experiments were performed. In one set of experiments, U2-OS cells were used as bystanders and MHC haplotype-matched T1, T2 or HMY-A2 cells served as antigen presenting cells pulsed with PEMA (Fig. 4). In the converse experiment, T1, T2 and HMY-A2 cells were used as bystanders and U2-OS cells were PEMA-pulsed (Fig. 5). Sensitization of bystander cells by excretion of peptide from PEMA-pulsed cells was not detected with any combination of cells. As positive controls, each bystander cell type was incubated with PEMA or peptide in the absence of other cells and shown to be sensitized for lysis by CTL. Therefore, it is unlikely that excretion of peptides accounts for the observed sensitization by PEMA.

To investigate potential internalization of MHC class I in U2-OS cells, surface levels were analyzed by immunofluorescence staining in the presence or absence of BFA. During the period of BFA treatment, cell number remained constant and cell viability, as measured by trypan blue exclusion, was not different from that of control cells (data not shown). After 10 h in BFA, cell surface MHC class I levels decreased to approximately 85% of the starting amount (Fig. 6A, B). Samples collected at intermediate time points showed that MHC class I molecules disappeared from the cell surface at a constant rate of  $\sim 1.5\%/h$  (Fig. 6C). This suggests that MHC class I underwent internalization, denaturation, and/or shedding from the cell surface with a half-time of approximately 33 h, which is comparable to that in other cells [34].



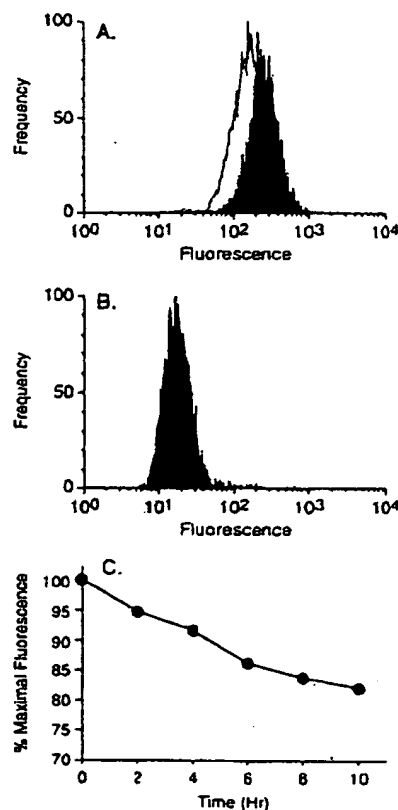
**Figure 5.** Lack of sensitization of T1, T2 and HMY-A2 cells by excretion of peptide from PEMA-pulsed U2-OS cells. T1 (panel A), T2 (panel B) and HMY-A2 (panel C) cells served as bystanders and were incubated with  $^{51}\text{Cr}$  overnight. U2-OS cells served as antigen presenting cells and were incubated with (+) or without (-) PEMA (1  $\mu\text{g}/\text{ml}$ ). Bystanders and PEMA-pulsed cells were incubated together and treated as described in Fig. 4. As positive controls, each bystander cell type was incubated with PEMA or peptide. Error bars represent  $\pm$  SD, where  $n = 3$ .

Taken together, these results: (i) demonstrate that intracellular trafficking of PEMA contrasts that of PE, in that sensitization of cells by PEMA does not require delivery from endosomes to the Golgi complex, ER or cytosol, (ii) demonstrate that sensitization by PEMA does not involve excretion of peptide for association with cell surface MHC class I, and (iii) suggest that U2-OS cell surface MHC class I molecules may undergo constitutive internalization.

#### 4 Discussion

The nature of antigen presentation by MHC class I has been well studied and it is clear that nascent MHC molecules in the ER associate with peptides derived from

endogenously synthesized antigens, such as viral proteins [25, 26]. However, it has been observed that, by using various vehicles, exogenous antigens can be delivered to the cytosol and presented by MHC class I molecules [10-13, 35, 36]. Exogenous proteins by themselves are generally not able to enter an MHC class I processing pathway. But, by utilizing fusion proteins consisting of the binding and translocating domains of PE, which has intrinsic capabilities to gain entry into intracellular compartments, we have succeeded in delivering exogenous proteins containing CTL epitopes to such a pathway. The key findings of this study are as follows: (i) the intracellular pathway taken by PEMA differs from that of PE at a point at or past endosomes, (ii) sensitization of target cells by PEMA does not require delivery of the matrix epitope to the cytosol, and (iii) association of the matrix epitope with MHC class I does not require newly synthesized MHC molecules. One might predict that delivery of PEMA to nascent MHC molecules in the ER would be inefficient, due to the lack of a KDEL-like sequence on the C-terminus of the fusion protein. Such a sequence on PE was recently



**Figure 6.** Cell surface staining of MHC class I. Cells were incubated with BFA (1  $\mu\text{g}/\text{ml}$ ) for up to 10 h and aliquots of cells were taken for immunofluorescence determination of cell surface MHC class I levels. Relative fluorescence intensity is plotted on the abscissa versus frequency on the ordinate for cells treated with BFA for 10 h (open peak) and untreated control (closed peak). Staining with monoclonal HLA-A2.1 antibody is shown in panel A, and with isotype-matched control antibody in panel B. MHC class I levels did not change during this time period in untreated cells (not shown). Samples taken at the intermediate time points of 2, 4, 6 and 8 h were also analyzed and are plotted together with the 0 and 10 h time points as percent maximal fluorescence intensity versus time (panel C).

shown to be obligatory for intoxication [18] and implies the involvement of the KDEL receptor, which is localized in the ER and Golgi complex [37], in the delivery of the toxin to the ER for translocation to the cytosol [19].

Taken together, the results in this report suggest that the matrix epitope was able to associate with preexisting MHC class I molecules in an endocytic compartment, such as could occur by the internalization of cell surface MHC class I. The generation of peptides for association with MHC class I could be the result of proteolytic processing of PEMA in endosomes. In support of this possibility, we have isolated fragments of PEMA from endosomes of cells incubated with radiolabelled PEMA (unpublished observations). Subsequent presentation of the matrix epitope in association with MHC class I could conceivably occur by recycling of the complex to the cell surface. These possible events remain to be established; however several published reports support the hypothesis. Endocytosis and recycling of MHC class I molecules to the cell surface has been described in activated T lymphocytes [38] and T cell lines [39, 40], and may be involved in antigen presentation [41], but it is not certain whether this can be generalized to other cell types as an ongoing process.

Internalization of MHC class I molecules has been reported to occur constitutively in T lymphocytes [42], macrophage/monocyte cells [43] and T and B lymphoid cell lines [44], and can be induced in fibroblasts by cross-linking [45]. Regulated internalization was also suggested in some cases, as evidenced by phorbol ester-induced enhancement of uptake [43, 44]. Incubation of P815 cells with a photoaffinity-labeled CTL epitope resulted in specific binding to MHC class I, some of which was subsequently localized intracellularly [46], suggesting that MHC class I molecules were spontaneously internalized. In addition, it has been proposed that the virus-induced disappearance of cell surface MHC class I molecules in BGIMK primate cells is caused by an impairment of normal MHC class I recycling [47]. Finally, two recent reports suggest the involvement of endolysosomal processing of antigens for presentation by MHC class I. First, CTL-mediated lysis of Sendai virus-infected T2 cells was not inhibited by BFA treatment [48], indicating that an alternative pathway can function in the presentation of antigens by MHC class I. Second, bacterial antigens were internalized, processed in a phagocytic compartment and presented by MHC class I molecules in BFA- and cycloheximide-treated cells [49]. In that study, it was postulated that intracellular antigen processing and excretion of peptide led to the expression of peptide-MHC complexes on the cell surface. However, the results could also be explained by internalization and recycling of cell surface MHC class I. In the present study, excretion of the matrix epitope by PEMA-pulsed cells was not detected, as measured by the lack of sensitization of haplotype-matched bystander cells.

In contrast to the cited examples of MHC class I internalization and recycling, internalization of MHC class I molecules in the JY human B lymphoblastoid cell line [50, 51] and in B lymphocytes [42] was not detected. Therefore, this process may depend on cell type or may be more prominent in some cells than others (for review see [52]). The results presented in this report, though, suggest that MHC class I molecules may not only be internalized to an endocytic

compartment but also may associate with antigenic peptides therein and recycle back to the plasma membrane in non-lymphoid cells, where they can be recognized by appropriate CTL. Such a pathway could explain why certain exogenously applied proteins without an apparent means of gaining access to the cytosol are capable of being presented by MHC class I molecules *in vitro* and/or inducing a CTL response *in vivo* [53-59]. That exogenous proteins in general do not enter this processing pathway suggests there is limited access. However, the use of ligands, such as PE, to target exogenous antigens containing CTL epitopes for endocytosis to intracellular processing compartments may facilitate their presentation by MHC class I, and may form the basis of vaccine efforts designed to generate CTL responses. To provide determinant selection in an outbred population, however, it will be necessary to first determine whether larger polypeptides can be targeted to cells and processed for presentation by MHC class I using this technique. Nevertheless, because of the sensitivity of CTL in recognizing antigen/MHC class I complexes [60], presentation of exogenous toxin/antigen fusion proteins by a putative recycling MHC class I pathway has potential as a means of eliciting CTL responses *in vivo*.

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## Targeting HIV proteins to the major histocompatibility complex class I processing pathway with a novel gp120-anthrax toxin fusion protein

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**ABSTRACT** A challenge for subunit vaccines whose goal is to elicit CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) is to deliver the antigen to the cytosol of the living cell, where it can be processed for presentation by major histocompatibility complex (MHC) class I molecules. Several bacterial toxins have evolved to efficiently deliver catalytic protein moieties to the cytosol of eukaryotic cells. Anthrax lethal toxin consists of two distinct proteins that combine to form the active toxin. Protective antigen (PA) binds to cells and is instrumental in delivering lethal factor (LF) to the cell cytosol. To test whether the lethal factor protein could be exploited for delivery of exogenous proteins to the MHC class I processing pathway, we constructed a genetic fusion between the amino-terminal 254 aa of LF and the gp120 portion of the HIV-1 envelope protein. Cells treated with this fusion protein (LF254-gp120) in the presence of PA effectively processed gp120 and presented an epitope recognized by HIV-1 gp120 V3-specific CTL. In contrast, when cells were treated with the LF254-gp120 fusion protein and a mutant PA protein defective for translocation, the cells were not able to present the epitope and were not lysed by the specific CTL. The entry into the cytosol and dependence on the classical cytosolic MHC class I pathway were confirmed by showing that antigen presentation by PA + LF254-gp120 was blocked by the proteasome inhibitor lactacystin. These data demonstrate the ability of the LF amino-terminal fragment to deliver antigens to the MHC class I pathway and provide the basis for the development of novel T cell vaccines.

Whereas serum IgG are believed to be sufficient for protection conferred by most current vaccines (1), CD8<sup>+</sup> cytotoxic T cells (CTLs) are believed to be important in clearing viral infections (1-8). A more vigorous CTL response against a viral infection thus should be beneficial (9-13). Thus, vaccines eliciting specific CTLs should be valuable, especially in the case of viruses that produce chronic infections, such as HIV. Although live viruses elicit CTL immunity, soluble whole proteins, such as subunit vaccines, with or without adjuvants, generally do not (14-17). Because whole exogenous proteins are usually internalized via the endosomal system, they are processed for presentation by class II major histocompatibility complex (MHC) molecules, generally not class I. In contrast, presentation by class I MHC molecules typically occurs only for proteins proteolytically processed in the cytoplasm of the cell. Cytosolic proteins are digested into short peptides by proteasomes, and the resulting peptides are transported by transporters of antigenic peptides into the endoplasmic reticulum,

where they bind to nascent class I MHC molecules, which carry them out to the cell surface for display to CD8<sup>+</sup> T cells (17). Therefore, CD8<sup>+</sup> CTLs, which uniquely recognize peptide epitopes presented by class I MHC molecules, provide surveillance against abnormal proteins synthesized in the cell, such as viral proteins or tumor antigens. The challenge in designing a nonliving vaccine for induction of CD8<sup>+</sup> CTLs thus is to introduce the vaccine protein into the cytosol of cells for processing and presentation by their class I molecules.

Several CTL vaccine strategies have been employed with varying effectiveness, including live attenuated bacterial and viral strains, immunostimulatory complexes (ISCOMs) and liposome delivery systems for soluble antigens, and DNA-based vaccines. Unfortunately, each of these systems contains its own unique set of problems and is not always suitable for all individuals (18-21). Particularly, in the case of HIV-1 and its macaque close cousin simian immunodeficiency virus (SIV), live attenuated virus has been shown to protect adult monkeys from a subsequent challenge with virulent virus (22), but the safety of an attenuated strain, especially in newborns, has been questioned (23).

A number of bacterial toxins contain catalytic domains that share the ability to gain access to the host cell cytosol, where they can exert their effects. Although each toxin may differ in the mechanism or route by which it gains entry to the cytosol, the overall effect is that of a "molecular syringe" that is able to inject the toxic protein into the cell. Several bacterial toxins, including diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), pertussis toxin, and the pertussis adenylate cyclase, have been used in attempts to deliver peptide epitopes to the cell cytosol as internal or amino-terminal fusions (24-27). These systems are restricted in their use as potential vaccines, because their capacity to deliver larger protein antigens is limited and many individuals have already been immunized against the carrier toxin.

Although peptides are able to stimulate a cellular immune response, whole protein antigens may be better suited for use in an effective vaccine for two reasons. First, the epitope that is essential for protection in one genetic background may prove to be irrelevant in a different genetic background. Therefore, it is beneficial for a broadly applied T cell vaccine to use the full-length protein from which the various relevant epitopes are derived. Second, peptides recognized by CTLs are processed from the whole protein by specialized degradative machinery, including the proteasome complex. In certain

Abbreviations: LF, lethal factor; PA, protective antigen; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; PE, *Pseudomonas* exotoxin A; GST, glutathione S-transferase.

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instances, the processing of the relevant peptide epitopes is dependent on the flanking amino acid sequences (28). However, flanking residues are not always important for proper processing (29). Because it currently is not possible to accurately predict which epitopes are dependent on their context for proper processing, it is important to deliver the entire antigen to the cell cytosol for optimal processing and presentation.

*Bacillus anthracis* secretes two toxins into the extracellular medium during growth (30, 31). The two toxins consist of three distinct proteins that combine in a pairwise fashion (32, 33). Protective antigen (PA) combines with lethal factor (LF) or edema factor (EF). PA combined with LF constitutes lethal toxin, whereas PA combined with EF makes edema toxin (34, 35). In a simple model of intoxication, PA (83 kDa) binds to a protein receptor on the surface of cells. PA is then cleaved by a cellular protease (furin) and the amino-terminal 20-kDa fragment is released, leaving a 63-kDa fragment, PA63, bound to the cell (36–38). PA63 is able to bind either LF or EF. The toxin is then endocytosed and transported into the cell. Conversion of PA to PA63 allows formation of an oligomeric form of PA, which, after exposure to low pH in late endosomes, forms channels in cell membranes (39, 40). It is believed that these channels facilitate the delivery of LF or EF from the endosome to the cytosol of the cell (41, 42). We have previously described the internalization of fusion proteins containing anthrax toxin lethal factor and the catalytic domains of other bacterial toxins (43–45). These active fusions were used to define the amino-terminal 254 aa of LF as those necessary for uptake of the fusions (46). In this work, we have taken advantage of the efficient delivery of LF fusion proteins to the cytosol to intracellularly inoculate living cells with whole protein antigens. This system will provide the basis for new potent CTL vaccines.

## MATERIALS AND METHODS

**Reagents and General Procedures.** Restriction endonucleases and DNA modifying enzymes were purchased from Life Technologies, Boehringer Mannheim, or New England Biolabs. Oligonucleotides were synthesized on a PCR mate (Applied Biosystems) and purified on oligonucleotide purification cartridges (Applied Biosystems). The PCR was performed with a GeneAmp kit according to the manufacturer's directions (Perkin-Elmer/Cetus). Bacterial media preparation, restriction digests, ligation, and phosphatase treatment of DNA were performed by standard protocols (47). Peptide P18IIIB was made by an automated peptide synthesizer (Applied Biosystems) and purified by HPLC liquid chromatography before use (48). Lactacystin was synthesized in the lab of E. J. Corey (Harvard University, Cambridge, MA) and was a generous gift of Jonathan Yewdell and Jack Bennink (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

**Plasmid Construction.** The plasmid used for expressing the LF254-gp120 fusion protein in *Escherichia coli* was constructed by ligation of the pGEX-KG vector (Pharmacia) with PCR-amplified LF and gp120 gene sequences. The DNA encoding residues 1–254 of LF were amplified from plasmid pLF7 with primers that added unique *Xba*I and *Mlu*I sites on the 5' and 3' ends, respectively (49). The primers were 5'-TCT AGA TCT AGA AGC GGG CGG TCA TGG TGA TGT AGG-3' (primer 1) and 5'-GAT CTT TAA GTT CAC GCG TGG ATA GAT TTA TTT CTT G-3' (primer 2). The gene for gp120 was amplified from plasmid HXB2-env with primers that added unique restriction sites for *Mlu*I on the 5' end and *Xho*I on the 3' end of the amplified gene (43). The sequences were 5'-CCG CGT ACG CGT ATG AGA GTG AAG GAG AAA TAT CAG-3' (primer 3) and 5'-TTC GAG CTC GAG TTA TCT TTT TTC TCT CTG CAC CAC-3' (primer 4). Primer 4 introduced a stop sequence (TAA) after the gp120

coding sequence. The amplified DNA products and the pGEX-KG plasmid DNA were digested with the appropriate restriction enzymes. Vector DNA was dephosphorylated with bacterial alkaline phosphatase for 30 min. All three DNA fragments were purified from low-melting-point agarose after electrophoresis by extraction with phenol-chloroform, mixed, and ligated overnight at 16°C with T4 DNA ligase. The ligated DNA was used to transform chemically competent *E. coli* (DH5 $\alpha$ , high efficiency, Life Technologies). Transformed *E. coli* were selected on ampicillin containing solid media (50  $\mu$ g/ml) and screened by restriction analysis of extracted plasmid. Clones that had the expected restriction pattern were confirmed by DNA sequencing.

**Expression and Purification of the LF254-gp120 Fusion Protein.** The use of plasmid pGEX-KG allowed for the expression of the three-part fusion protein (GST-LF254-gp120) after induction by isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) and rapid purification by adsorption to glutathione-Sepharose 4B. The expression and purification of GST-LF fusion proteins was described previously (44). *E. coli* strain SG12036 was transformed with the pGEX-KG-LF254-gp120 fusion vector and grown in rich media (superbroth, 100  $\mu$ g/ml of ampicillin) with shaking at 225 rpm at 37°C. When the cell density at A<sub>600</sub> reached 0.6–0.8, IPTG was added to a final concentration of 1 mM. After further incubation for 2 hr, the bacterial cells were pelleted by centrifugation and then resuspended in 100 mM phosphate buffer (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml 4-(2-aminoethyl)-benzenesulfonylfluoride. The bacterial cells were disrupted by sonication, and the clarified extracts were applied to a glutathione-Sepharose 4B column previously equilibrated with buffer (100 mM phosphate, pH 7.4/150 mM NaCl/1% Triton X-100). The column was washed extensively, and the bound fusion protein was eluted with 10 mM glutathione in 50 mM Tris, pH 8.0/0.5 mM EDTA. The eluted protein was concentrated by ultrafiltration with a Centriprep-30 device (Amicon) and analyzed for purity by electrophoresis on nondenaturing and SDS polyacrylamide gels (Phast gels, Pharmacia). Protein concentrations were determined by the micro BCA method with BSA as a standard (Pierce).

**Protective Antigen Proteins.** Protective antigen was expressed in *B. anthracis* from the expression vector pYS5 and purified by established procedures (50, 51). Mutant PA molecules PA CFD and PA-D were constructed by site-directed mutagenesis and have been previously described (52).

**Cell Lines.** P815, a DBA/2-derived (H-2<sup>d</sup>) mastocytoma (ATCC TIB-64) used as target cells in the CTL assay, was maintained in RPMI 1640 medium supplemented with 10% FCS. The HIV gp120-specific CTL lines 9.23.3 and  $\alpha$ 15-12, which recognize the V3 epitope RGPGRFVTI, have been previously described (13, 48, 53). HIV gp120-specific CTL lines were derived from BALB/c spleens taken from mice previously immunized with a recombinant vaccinia virus expressing the gp160 protein and restimulated weekly. 9.23.3 CTLs were stimulated with 10  $\mu$ M P18IIIB peptide pulsed on  $5 \times 10^6$  irradiated splenocytes [3,000 rads (1 rad = 0.01 Gy)] with  $5 \times 10^5$  CTLs per well, in a 24-well plate containing 2 ml of a 1:1 mixture of RPMI 1640 medium and Eagle-Hanks' amino acid medium (EHAA) supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10% fetal calf serum, and 10% T-stim (Collaborative Biomedical Products). Stimulation of the  $\alpha$ 15-12 CTL line used the same media mixture; however, these CTLs were restimulated with mitomycin C-treated  $\alpha$ 15-12 cells at  $5 \times 10^5$  CTLs,  $5 \times 10^5$  15-12 cells, and  $5 \times 10^6$  irradiated (3,000 rads) BALB/c splenocytes per well. 15-12 cells were isolated from transfected BALB/c 3T3 cells, which express the gp160 protein from the IIIB strain of HIV-1 (48).

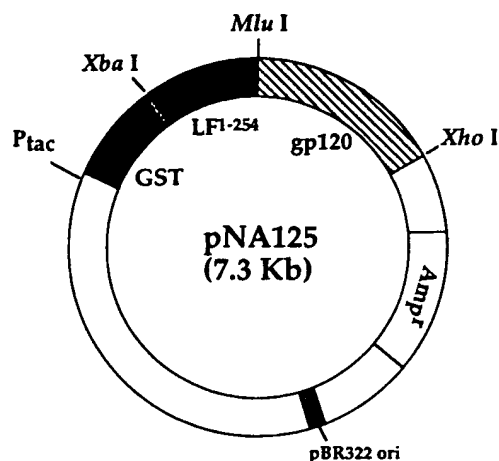
**Cytotoxic Lymphocyte Assay.**  $^{51}\text{Cr}$  release assays were performed as previously described (54). When appropriate, target cells were treated with PA (100 ng/ml) and/or LF254-gp120 fusion protein (1.0 ng/ml when used with  $\alpha 15-12$  CTL or 50 ng/ml when used with 9.23.3 CTL) for 12 hr in serum-free media. After treatment, the target cells ( $5 \times 10^5$  cells) were labeled with 200  $\mu\text{Ci}$  (1 Ci = 37 GBq) of  $\text{Na}_2^{51}\text{CrO}_4$  in 100  $\mu\text{l}$  of RPMI 1640 for 2 hr at  $37^\circ\text{C}$ . In some cases, target cells were pulsed with peptide (1.0–10.0  $\mu\text{M}$ ) during labeling. Following labeling, targets were washed three times and added at 3,000 cells per well along with the appropriate number of effector cells in 96-well round-bottom plates. Supernatants were harvested after 6 hr and counted in an Isomedic gamma counter (ICN). The mean of triplicate samples was calculated and the percent  $^{51}\text{Cr}$  release was determined using the following formula. Percent  $^{51}\text{Cr}$  release =  $100 \times [(\text{experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}) / (\text{maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release})]$ , where experimental  $^{51}\text{Cr}$  release represents counts from target cells mixed with effector cells, control  $^{51}\text{Cr}$  release represents target cells mixed with medium alone (spontaneous release), and maximum  $^{51}\text{Cr}$  release represents counts from target cells exposed to 2.5% Triton X-100.

## RESULTS

**Plasmid Construction.** The LF sequences required for binding to PA and translocation to the cell cytosol were genetically fused to the gp120 portion of the envelope gene from HIV-1 strain HXB2. This fusion was constructed in the pGEX-KG vector, resulting in a three-way protein fusion of the 26-kDa glutathione S-transferase domain with LF residues 1–254 and residues 1–511 of the gp160 protein. The inclusion of the Gly<sub>5</sub>-containing linker from pGEX-KG and the alteration of a base following the *Xba*I site before the 5' end of the LF1–254 gene fragment resulted in a modified 14-residue spacer (GSPGISGGGGGILE) between the GST domain and LF1–254 (Fig. 1). The addition of the unique *Mlu*I and *Pst*I sites to the 5' end of the coding sequences for the gp120 portion of the envelope gene added four additional residues, TRLQ, between LF1–254 and gp120 (Fig. 1). The addition of residues at the amino- and carboxyl-terminal ends of LF1–254 do not appear to affect the function of the protein with respect to PA binding and protein translocation (42, 44). The resulting fusion protein has a calculated molecular mass of 114,852 Da and a calculated pI of 7.00.

**Specific Cytolysis of Target Cells Sensitized with LF254-gp120 Fusion Proteins by HIV gp120-Specific CTL.** The mouse mastocytoma cell line P815 was incubated with PA or PA mutants and/or LF254-gp120 fusion protein for 12 hr in serum-free conditions. The cells were then washed and labeled with  $^{51}\text{Cr}$  for use as target cells. The labeled P815 target cells were mixed at different ratios with the effector CTL cell line 9.23.3, which recognizes the peptide epitope, RGPGRFVTI, from the V3 loop of gp120 (13, 48, 53). Killing of the target cell population was determined by measuring release of  $^{51}\text{Cr}$  into the media (Fig. 2). Target cells treated with wild-type PA and LF254-gp120 fusion protein were efficiently recognized and lysed by the 9.23.3 CTL line. To ensure maximal lysis, effector-to-target ratios starting at 40:1 were used. Lysis was still on a plateau at an effector-to-target ratio of 10. The recognition and lysis was dependent on the presence of functional PA, demonstrating that processing of the fusion protein relies on internalization via the PA receptor. Treatment with a translocation-defective mutant PA protein (52) or without the addition of PA resulted in minimal lysis of the target cells, placing a limit on the amount of LF254-gp120 that is proteolytically degraded extracellularly and thus able to sensitize target cells without active transport into the cytosol. This implies that the LF254-gp120 fusion protein needs to gain access to the cell cytosol for processing and presentation.

A.



B.

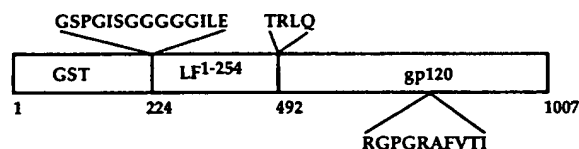


FIG. 1. Plasmid construct and fusion protein. (A) Plasmid NA125 construct for expression of LF254-gp120 fusion protein in *E. coli*. GST, 26-kDa glutathione S-transferase domain from the parent vector pGEX-KG; LF1–254, coding sequences for residues 1–254 of mature LF; gp120, coding sequences for residues 1–511 of HXB2-env gene. (B) Expressed GST-LF254-gp120 protein. The carboxyl-terminal residue of the linker from pGEX-KG was changed from D to E, and four residues, TRLQ, were added between the LF254 and gp120 portions of the construct due to DNA manipulations. The V3 epitope, RGPGRFVTI, recognized by CTL begins at residue 807 of the fusion protein.

Treatment with PA alone, mutant PA alone, or the LF254-gp120 fusion alone did not sensitize the target cells for lysis. Target cells pulsed with P18IIB peptide served as a positive control and resulted in cell lysis when effector cells were added. To compare the relative potency of the LF254-gp120 with that of the optimal decapeptide P18-I10 for sensitizing target cells for recognition by these CTL (53, 55, 56), titration experiments were performed. The P18-I10 decapeptide sensitized targets for lysis in the range of  $10^{-10}$  to  $10^{-11}$  M, based on specific lysis  $>10\%$  as a threshold for positivity, whereas the LF254-gp120 sensitized targets with as little as  $4 \times 10^{-14}$  M protein (data not shown). Therefore, the fusion construct was at least 2.5 logs more potent on a molar basis than the optimal binding peptide.

**Lactacystin Prevents Lysis of PA/LF254-gp120-Treated Target Cells.** To demonstrate the requirement for LF254-gp120 processing by the classical MHC class I pathway, we examined the ability of the specific proteasome inhibitor, lactacystin, to inhibit presentation of the V3 epitope on the cell surface. Incubation of P815 cells with 10  $\mu\text{M}$  lactacystin for 45 min prior to the addition of PA and LF254-gp120 significantly decreased lysis of the target cells by the  $\alpha 15-12$  CTL line (Fig. 3). Lactacystin inhibition of peptide presentation shows that the processing of the peptide epitope from the fusion protein depends on the function of the proteasome complex. This indicates that the protein fusion was delivered to the cytosol of the cell, where it interacts with the proteasome complex and precludes the role of any alternate processing pathways for presentation by the anthrax toxin LF254-gp120 fusion protein.

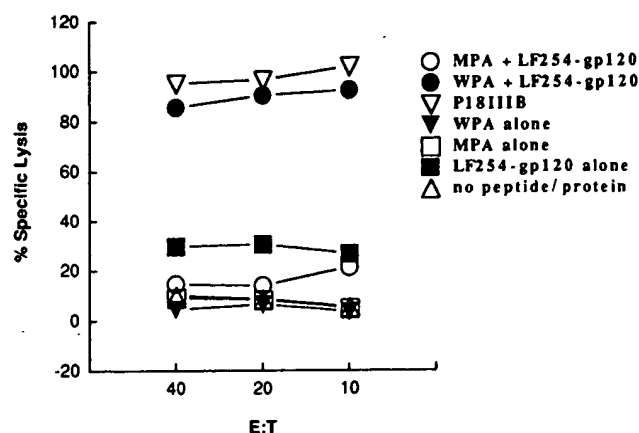


FIG. 2. Recognition of cells treated with gp120 anthrax toxin fusions by gp120-specific cytotoxic lymphocytes. The mouse mastocytoma cell line P815 was incubated with wild-type protective antigen (WPA) or mutant PACFD (MPA) and/or LF254-gp120 fusion proteins (50 ng/ml). P815 cells pulsed with 1.0  $\mu$ M P18111B peptide served as a control. After 12 hr, the cells were washed and the treated P815 cells were labeled with  $^{51}$ Cr. Labeled P815 cells were then mixed at different ratios with the CTL cell line 9.23.3, which recognizes the peptide sequence RGPGRFVFI from gp120. Killing of the target cell population was determined by measuring release of  $^{51}$ Cr into the medium (% lysis). Background lysis was determined with untreated,  $^{51}$ Cr labeled, P815 cells. These data are the replicate of three experiments.

## DISCUSSION

The primary goal of this study was to determine the ability of an anthrax lethal factor fusion to deliver antigenic proteins to the cell cytosol for processing and presentation with class I molecules on the cell surface. We demonstrated the ability of the LF254-gp120 fusion, in conjunction with PA, to sensitize target cells (Fig. 2). The requirement for functional PA shows that the LF254-gp120 fusion needs to gain access to the intracellular environment to be processed. Other toxins that have been used as fusions with peptide epitopes have also resulted in presentation of the epitope on the surface of antigen presenting cells in the proper context. Examples include DT, PE, pertussis toxin, and the *Bordetella pertussis* adenylate cyclase (24–27, 57). However, the processing and presentation of peptide epitopes from each of these fusion proteins do not appear to occur through the classical MHC class I processing pathway but instead via various alternate pathways.

DT was used as a delivery system with peptide epitopes from influenza virus fused to the amino-terminal end of the toxin (24). Although it was demonstrated that the peptide-toxin fusion gained access to the inside of the cell, processing and presentation of the antigens was not demonstrated. Subsequently, it was reported that DT was able to translocate additional protein domains into the cell cytosol (57, 58). Genetic fusions of an inactive DT protein with an active DT catalytic domain were translocated into the cytosol, where they were toxic. However, fusions with other protein domains had reduced abilities to deliver proteins or were unable to bind to the DT receptor and were not translocated. The entire genetic fusion appeared to be very stable in the cytosol, with no indication of any processing 6 hr after translocation, which may imply that the translocated DT or DT fusions are not accessible to the degradative machinery in the cell.

A truncated PE has also been genetically fused to influenza A peptide epitopes (25). The ability of PE-peptide antigen fusions to deliver the antigenic peptide to the MHC class I molecules on the surface of target cells was examined. Specific lysis by CTLs recognizing the influenza A epitopes within the

fusion protein was detected. However, it was determined that the PE fusions trafficked through the cell differently from native PE. In fact, blocking translocation after adding the PE fusions to cells had no effect on processing and presentation of the antigenic peptide (59). The authors proposed that a certain amount of proteolysis occurs in the endosome and that there exist, in the endosome, MHC class I molecules able to bind the processed peptide; thus, recycling of these complexes to the cell surface can mark the cell for recognition and lysis by CTLs. These PE fusions may enter a noncytosolic pathway for presenting exogenous antigens to the MHC class I molecules (60, 61).

Peptides from the lymphocytic choriomeningitis virus nucleoprotein were inserted into the S1 subunit of pertussis toxin and the pertussis adenylate cyclase (26, 27, 57, 62). These fusions specifically sensitized target cells to the appropriate CTLs. However, the dependence on the classical pathway for processing and presentation of the antigen to the MHC class I pathway was not established. The adenylate cyclase fusions were tested for their effectiveness *in vivo* and were able to generate CTLs. Production of CTLs was dependent on coinjection of the fusion protein with alum, which implies that the fusions are processed by an alternate exogenous pathway for MHC class I antigen.

In the case of LF254-gp120, we have demonstrated that delivery to the cytosol requires active PA and that presentation requires a functional proteasome complex. Use of lactacystin, a specific inhibitor for proteasomes, provides an unequivocal demonstration that these constructs enter the cytosolic degradative pathway involved in classical processing for class I MHC presentation (Fig. 3) (63). Thus, in this case a bacterial toxin fusion protein has been definitively shown to deliver a whole protein antigen to the classical cytosolic MHC class I pathway.

The anthrax lethal toxin system has several additional advantages over the other bacterial toxins for vaccine use. First, LF fusion proteins are nontoxic, because the entire catalytic domain has been deleted. Even the "detoxified" DT still retains 1/800 of the wild-type activity, which is enough to preclude its use *in vitro* or *in vivo* (24, 64). In contrast, the entire catalytic domain of LF, residues 255–776, has been deleted in LF254-gp120. Our prior studies have shown that only LF residues 1–254 are needed for efficient delivery of fused polypeptides (46). Second, LF fusions can be produced as soluble proteins in standard GST expression vectors. The binary nature of the toxin allows production of LF fusions without manipulation or alteration of the cell-binding properties of PA. LF residues 1–254 appear indifferent to addition of peptides or larger proteins at either the amino or carboxyl termini and still retain their ability to bind PA (44). These additions to LF do not appear to seriously affect the ability of PA to translocate protein into the cell cytosol. In contrast, expression and purification of the other toxin fusions require either use of special hosts capable of secretion of the modified holotoxins or refolding after purification from inclusion bodies. Third, LF fusions appear to be proficient at delivering proteins to the cell cytosol. LF-*Pseudomonas* exotoxin A fusions are 100 times more potent than wild-type PE, demonstrating that the delivery of the active domain is more efficient (43). The ability of our system to efficiently deliver antigen to the cytosol is reflected in the comparatively small amount of protein needed to achieve recognition by CTL. Both the pertussis cyclase delivery system and the pertussis toxin delivery system require 50 times more antigen on a molar basis to achieve similar recognition of target cells by CTL (26, 27). Finally, LF254 appears capable of delivering protein antigens that are large enough that hosts from varied genetic backgrounds can be expected to process the appropriate epitope from the antigen and properly present it.

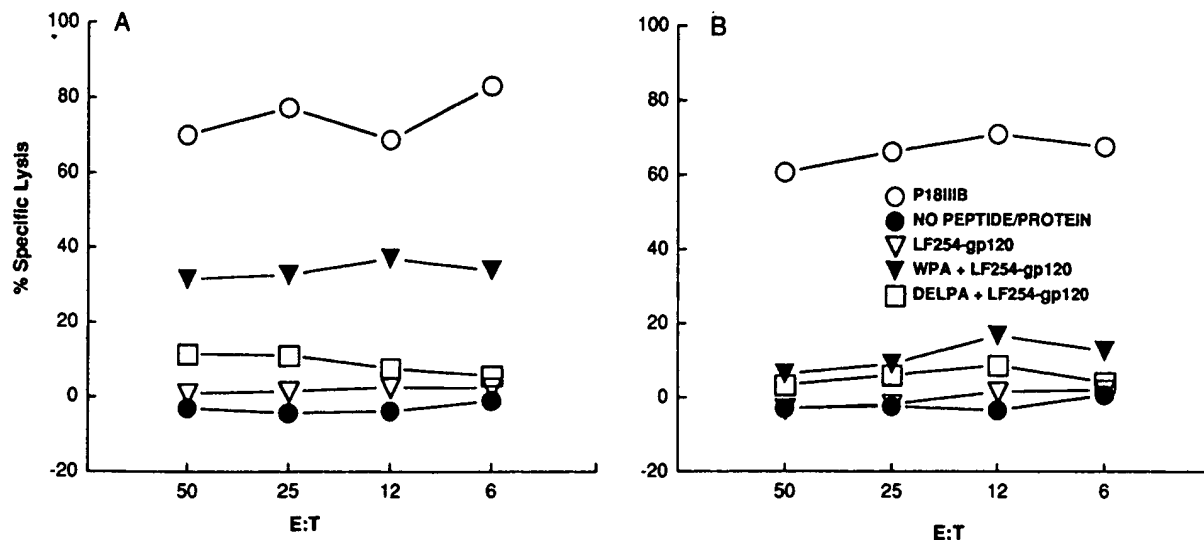


FIG. 3. Inhibition of fusion protein processing by lactacystin. P815 cells were treated with 1 ng/ml of the GST-LF254-gp120 fusion protein alone and with PA (WPA) or mutant PA-D (DELPA) in the presence (B) or absence (A) of 10  $\mu$ M lactacystin.  $^{51}$ Cr-labeled target cells were mixed with  $\alpha$ 15-12 CTLs, and specific lysis was measured. Target cells were treated with the P18IIIB peptide as a positive control for cell lysis. These data are the representative of three experiments.

Preliminary *in vivo* experiments show that a single subcutaneous injection of the LF254-gp120 fusion along with PA is able to elicit a vigorous CTL response in the spleens of vaccinated animals (data not shown). We are currently refining the immunization procedure and testing the PA dependence of the *in vivo* response to confirm a cytosolic processing mechanism, as well as investigating the ability of other LF254-antigen fusions, including LF254-gp41, LF254-reverse transcriptase, and LF254-NEF, to generate an immune response.

A recent study by Ballard *et al.* (65), performed concurrently with the present study, addresses the effectiveness of *in vivo* immunization with anthrax toxin fusion constructs, using a fusion of LF with a nine-residue peptide from the listeriolysin protein of *Listeria monocytogenes*, and shows protective efficacy against infection. However, because this study used only a nonpeptide minimal fragment from the protein, which can bind directly to class I MHC molecules and does not require processing through the class I pathway, it did not address the question of whether the toxin fusion construct could introduce a whole foreign vaccine protein into the cytosolic class I MHC processing pathway and elicit appropriate degradation and presentation with a class I MHC molecule. Our present study demonstrates this critical mechanistic point and shows that whole protein antigens may be introduced in this fashion for potential use as vaccines. Whole protein antigens have the advantage that they are not limited to single epitopes that are presented by only one or a few MHC molecules and are also more susceptible to escape mutations. However, they have the disadvantage that they are usually harder to introduce into the class I MHC processing pathway without a live viral vector (17). Thus, the major advantage of anthrax toxin fusion proteins is the ability to introduce whole complex foreign proteins to this processing pathway. Thus, the Ballard *et al.* study (65) and ours complement each other in demonstrating the utility of the anthrax lethal toxin fusion system for the stimulation of a cellular immune response.

In conclusion, we have taken advantage of several unique properties of anthrax toxin to construct fusion proteins that transport large proteins into the cytosol of living cells, where they are processed through the classical pathway for loading onto MHC class I molecules and presentation to CD8 CTL. Anthrax toxin appears particularly well suited, of all the bacterial toxins, to development of safe and potent vaccines for induction of CTL immunity.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/937,276	09/15/1997	KURT KLIMPEL	15280-290100	1627

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03/08/2002

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EXAMINER

SCHWADRON, RONALD B

ART UNIT

PAPER NUMBER

1644

DATE MAILED: 03/08/2002

**Response Due** 6-8-02 MNR

Please find below and/or attached an Office communication concerning this application or proceeding.

## Office Action Summary

Application No.

08/937,276

Applicant(s)

Klimpel et al.

Examiner

Ron Schwadron

Art Unit

1644



-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

### Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136 (a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

### Status

- 1) ☒ Responsive to communication(s) filed on 8/30/2001 and 12/17/2001.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

### Disposition of Claims

- 4) ☒ Claim(s) 9-19 is/are pending in the application.
- 4a) Of the above, claim(s) \_\_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 9-19 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_\_ is/are objected to.
- 8) ☐ Claims \_\_\_\_\_ are subject to restriction and/or election requirement.

### Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on \_\_\_\_\_ is/are objected to by the Examiner.
- 11) ☐ The proposed drawing correction filed on \_\_\_\_\_ is: a) ☐ approved b) ☐ disapproved.
- 12) ☐ The oath or declaration is objected to by the Examiner.

### Priority under 35 U.S.C. § 119

- 13) ☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).
- a) ☐ All b) ☐ Some\* c) ☐ None of:
- ☐ Certified copies of the priority documents have been received.
  - ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\*See the attached detailed Office action for a list of the certified copies not received.

- 14) ☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

### Attachment(s)

- 15) ☐ Notice of References Cited (PTO-892)
- 16) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 17) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s). \_\_\_\_\_
- 18) ☐ Interview Summary (PTO-413) Paper No(s). \_\_\_\_\_
- 19) ☐ Notice of Informal Patent Application (PTO-152)
- 20) ☐ Other:



1. Claims 9-19 are under consideration. Claims 1-7,20-28 have been canceled.

## RESPONSE TO APPLICANTS ARGUMENTS

2. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

3. Claims 9-19 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for the claimed invention which uses an APABP construct which contains amino acids 1-254 of LF is not enabling for the claimed invention which uses intact LF. Arora et al. (1993) discloses that intact LF causes cell death when administered to cells (see page 3334, first column). Thus, the claimed invention which uses intact LF could not be used to induce an immune response to CTL epitopes because it would kill the treated cells. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

4. Claims 9-19 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 9 is indefinite in the recitation of "anthrax protective antigen binding protein". The specification page 7, lines 1 and 2, defines said term as "contains the PA binding site of LF". The specification page 7, lines 21-23 defines LF as a 90 kDa protein derived from *B. anthracis*. Said protein is known in the art. The indefinite issue arises in that page 7, lines 23-24 recites "The description of LF includes binary toxin functional equivalents such as protein Ia of *C. perfringens*.". It is unclear from this sentence whether LF is limited to the 90 kDa protein derived from *B. anthracis* or whether LF encompasses includes binary

toxin functional equivalents because it is unclear whether "The description of LF includes" refers to the definition of LF or has some other meaning. If the term is taken as encompassing "binary toxin functional equivalents", then it is unclear what this term means or encompasses because it is not defined in the specification and it has no art recognized meaning. It is unclear as to what properties or set of properties would be encompassed by a "binary toxin functional equivalent".

5. Claims 9-19 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The specification does not provide adequate written description of the claimed invention. The legal standard for sufficiency of a patent's (or a specification's) written description is whether that description "reasonably conveys to the artisan that the inventor had possession at that time of the . . . claimed subject matter", *Vas-Cath, Inc. V. Mahurkar*, 19 U.S.P.Q.2d 1111 (Fed. Cir. 1991). In the instant case, the specification does not convey to the artisan that the applicant had possession at the time of invention of the claimed inventions.

As per paragraph 4 of the instant Office Action, instant claims encompass use of a "binary toxin functional equivalents". With the exception of the particular LF derived fragment derived from the first 254 amino acids of the 90 kDA protein of *B. anthracis* disclosed in the specification, the skilled artisan cannot envision the detailed structure of the encompassed "binary toxin functional equivalents" and therefore conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the method of isolation. For example, it is clear that the peptide recited in the claims encompasses a vast number of potential analogs with varying amino acid sequences, yet there is no disclosure in the specification of what amino acid sequence would be used other than the specific example disclosed in the specification. Adequate written description requires more than a mere statement that it is part of the invention and a reference to a potential method of isolating it. In the instant application, the amino acid itself or isolated protein is required. See *Fiers v. Revel*, 25 USPQ 2d 1601 at 1606 (CAFC 1993) and *Amgen Inc. V. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016.

In view of the aforementioned problems regarding description of the claimed invention, the specification does not provide an adequate written description of the invention claimed herein. See *The Regents of the University of California v. Eli Lilly and Company*, 43 USPQ2d 1398, 1404-7 (Fed. Cir. 1997). In *University of California v. Eli Lilly and Co.*, 39 U.S.P.Q.2d 1225 (Fed. Cir. 1995) the inventors claimed a genus of DNA species encoding insulin in different vertebrates or mammals, but had only described a single species of cDNA which encoded rat insulin. The court held that only the nucleic acids species described in the specification (i.e. nucleic acids encoding rat insulin) met the description requirement and that the inventors were not entitled to a claim encompassing a genus of nucleic acids encoding insulin from other vertebrates, mammals or humans, *id.* at 1240. The Federal Circuit has held that if an inventor is "unable to envision the detailed constitution of a gene so as to distinguish it from other materials. . .conception has not been achieved until reduction to practice has occurred", *Amgen, Inc. v. Chugai Pharmaceutical Co, Ltd.*, 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991). Attention is also directed to the decision of *The Regents of the University of California v. Eli Lilly and Company* (CAFC, July 1997) wherein is stated:

"The description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention. See *In re Wilder*, 736 F.2d 1516, 222 USPQ 369, 372-373 (Fed. Cir. 1984) (affirming rejection because the specification does "little more than outlin[e] goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate."). Accordingly, naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of, is not a description of that material. Thus, as we have previously held, a cDNA is not defined or described by the mere name "cDNA," even if accompanied by the name of the protein that it encodes, but requires a kind of specificity usually achieved by means of the recitation of the sequence of nucleotides that make up the cDNA." See *Fiers*, 984 F.2d at 1171, 25 USPQ2d at 1606.

7. The rejection of claims 9-19 under 35 U.S.C. 103(a) as being unpatentable over *Milne et al.* (*Mol. Microbiology* 15:651, 1995), *Arora et al.* (*J. Biol. Chemistry* 268:3334, 1993) or *Leppla et al.* US Patent 5,591,631 in view of EP 0 532 090A2 ( issued March

Art Unit: 1644

3, 1991) and Donnelly et al . (PNAS 90:3530:1993) is withdrawn in view of applicants arguments and the Berzofsky declaration filed 8/30/2001.

8. No claim is allowed.

9. Papers related to this application may be submitted to Group 1600 by facsimile transmission. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). Papers should be faxed to Group 1600 at (703) 308-4242.

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Dr. Ron Schwadron whose telephone number is (703) 308-4680. The examiner can normally be reached Monday through Thursday from 7:30 to 6:00. A message may be left on the examiners voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ms. Christina Chan can be reached on (703) 308-3973. Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is (703) 308-0196.



RONALD B. SCHWADRON  
PRIMARY EXAMINER  
GROUP 1600 *1600*

Ron Schwadron, Ph.D.

Primary Examiner

Art Unit 1644

## **Attachment for PTO-948 (Rev. 03/01, or earlier)**

6/18/01

**The below text replaces the pre-printed text under the heading, "Information on How to Effect Drawing Changes," on the back of the PTO-948 (Rev. 03/01, or earlier) form.**

### **INFORMATION ON HOW TO EFFECT DRAWING CHANGES**

#### **1. Correction of Informalities -- 37 CFR 1.85**

New corrected drawings must be filed with the changes incorporated therein. Identifying indicia, if provided, should include the title of the invention, inventor's name, and application number, or docket number (if any) if an application number has not been assigned to the application. If this information is provided, it must be placed on the front of each sheet and centered within the top margin. If corrected drawings are required in a Notice of Allowability (PTOL-37), the new drawings **MUST** be filed within the **THREE MONTH** shortened statutory period set for reply in the Notice of Allowability. Extensions of time may **NOT** be obtained under the provisions of 37 CFR 1.136(a) or (b) for filing the corrected drawings after the mailing of a Notice of Allowability. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftsperson.

#### **2. Corrections other than Informalities Noted by Draftsperson on form PTO-948.**

All changes to the drawings, other than informalities noted by the Draftsperson, **MUST** be made in the same manner as above except that, normally, a highlighted (preferably red ink) sketch of the changes to be incorporated into the new drawings **MUST** be approved by the examiner before the application will be allowed. No changes will be permitted to be made, other than correction of informalities, unless the examiner has approved the proposed changes.

#### **Timing of Corrections**

Applicant is required to submit the drawing corrections within the time period set in the attached Office communication. See 37 CFR 1.85(a).

Failure to take corrective action within the set period will result in **ABANDONMENT** of the application.

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